Complete developmental cycle of *Leishmania mexicana* in axenic culture

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SUMMARY

A complete developmental sequence of *Leishmania mexicana* has been produced in axenic culture for the first time. This was achieved by manipulation of media, pH and temperature conditions over a period of 16 days. All experiments were initiated with lesion amastigotes that were transformed to multiplicative promastigotes by culture in HOMEM, 10% foetal calf serum, pH 7·5, at 25 °C. Metacyclogenesis was induced by subpassage in Schneider's *Drosophila* medium, 20% foetal calf serum, pH 5·5, and the resulting forms transformed to axenically growing amastigotes by subpassage in the same medium and raising the temperature to 32 °C. Parasites from each day were characterized with respect to their general morphology using light microscopy of Giemsa-stained smears, and biochemically by analysis of total protein content, proteinases, nucleases and secretory acid phosphatase. The results demonstrated that the three main stages identified – amastigotes, multiplicative promastigotes and metacyclic promastigotes – each exhibited the expected suite of biochemical properties. Further, the changes in morphology observed as the developmental sequence proceeded from stage to stage were accompanied by appropriate changes in biochemical properties. These results provide both useful biochemical markers and a culture system in which to examine the regulation of differentiation and transformation during the *Leishmania* life-cycle.

Key words: *Leishmania*, axenic culture, amastigote, promastigote, metacyclic, proteinase, nuclease, acid phosphatase.

INTRODUCTION

Leishmania parasites exist in two hosts: as flagellated extracellular promastigotes in the alimentary canal of female phlebotomine sandflies and as intracellular amastigotes within phagolysosomes of mammalian macrophages. Transmission from sandfly to mammalian host is achieved by inoculation of a distinct mammal-infective form termed the metacyclic promastigote (Sacks, 1989; Killick-Kendrick, 1990). Metacyclics are phagocytosed by tissue macrophages and subsequently transform into intracellular amastigotes. These multiply within the phagolysosomal system, eventually rupturing the host cell, and the liberated amastigotes are taken up by other macrophages. Infection of a female sandfly is initiated if infected macrophages are ingested when taking a bloodmeal. Within the midgut freed amastigotes transform into promastigotes which multiply and establish an initial midgut infection. Subsequently, various morphological types of promastigotes have been observed during development in the sandfly host (Killick-Kendrick, 1990), although the precise relationships between these are unclear. Ultimately, however, metacyclic forms are produced in the anterior midgut and/or foregut and are responsible for transmission when the sandfly feeds again.

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There is evidence that biochemical differences exist between multiplicative promastigotes, metacyclic promastigotes and amastigotes (see, for example, Mallinson & Coombs, 1989; Rainey & MacKenzie, 1991; Robertson & Coombs, 1992; Schneider *et al.* 1992). These differences are likely to reflect the biochemical adaptations of each stage to its immediate environment and its particular role in the life-cycle. One of the major aims of current research is to further understand the molecular basis of adaptation and survival of each parasite stage. Determination of the conditions required for axenic culture has yielded much useful information on parasite biochemistry and cell biology. Promastigote cultures are relatively easy to obtain. They can be initiated by transformation of amastigotes *in vitro* and the resulting promastigotes maintained in long-term culture (reviewed by Evans, 1987). Axenic culture of amastigotes has proved more difficult (as these are an intracellular stage) but has been achieved for some species (reviewed by Bates, 1993*a*). Among other considerations, determination of the optimal temperature and pH conditions is important for both promastigote and amastigote culture. For example, promastigotes and amastigotes of *L. mexicana* can be cultivated axenically under conditions of 25 °C, pH 7·5 and 32 °C, pH 5·5, respectively (Hart, Vickerman & Coombs, 1981; Bates *et al.* 1992). Recently, a method for the cultivation of the metacyclic stage of *L. mexicana* at pH 5·5 was developed (Bates &

Tetley, 1993). In this report these culture methods have been further developed to reproduce, for the first time, a complete developmental sequence of *L. mexicana*. The resulting parasites have been characterized with respect to their morphology and various biochemical properties: protein content, nucleases and secretory acid phosphatase.

MATERIALS AND METHODS

Cell culture

Leishmania mexicana (MNYC/BZ/62/M379) was maintained in rump lesions of female CBA mice. Amastigotes were isolated and transformed to promastigotes as described (Hart *et al.* 1981; Bates *et al.* 1992) using HOMEM supplemented with 10% foetal calf serum and 25 μg gentamicin sulphate/ml. Late log phase cultures of recently transformed (3 days) promastigotes were subinoculated into Schneider's *Drosophila* medium supplemented with 20% foetal calf serum and 25 μg gentamicin sulphate/ml at pH 5·5 to induce metacyclogenesis (Bates & Tetley, 1993). After a further 6 days the resulting stationary phase populations were largely composed of metacyclic-like promastigotes. These were then used for transformation to amastigote-like forms by subpassage in the same medium at 32 °C. On each day, parasites were harvested from culture by centrifugation at 2000 ***g***, and 4 °C for 10 min, and washed by resuspension in serum-free medium and further centrifugation. Pellets were stored at −70 °C until required. Cell counting, cell measurements and light microscopy were performed as described (Bates & Tetley, 1993). Cell length measurements were made on a minimum of 40 cells and the results expressed as means ± standard deviations.

HOMEM was prepared essentially as described (Berens, Brun & Krassner, 1976) by dissolving powdered S-MEM (with Earle's Salts and L-glutamine, Gibco BRL Life Technologies 072-01400) in distilled water, then adding glucose (2 g/l), sodium bicarbonate (0·3 g/l), sodium pyruvate (0·11 g/l), *p*-aminobenzoic acid (1 mg/l), biotin (0·1 mg/l), HEPES (25 mM), MEM amino acids solution (10 ml/l of 50×, Gibco 043-01130), and MEM non-essential amino acids (10 ml/l of 100×, Gibco 043-01140). The pH was adjusted to 7·5–7·6 with NaOH pellets, the final volume adjusted with distilled water, and the HOMEM filter sterilized and stored at 4 °C. Schneider's *Drosophila* medium was purchased from Gibco BRL in liquid form (041-01720).

Parasites were classified into six categories based upon their morphology in Giemsa-stained smears. (i) Lesion amastigotes, which comprised 100% of the initial cell population in all transformation experiments. On subsequent days parasites of identical appearance which had not undergone any apparent increase in size were also classified as lesion amastigotes. (ii) Type I transformation intermediates. After inoculation of promastigote culture medium with lesion amastigotes various intermediate forms in the process of transformation from amastigotes to promastigotes were observed (see Hart *et al.* 1981 for a fuller description). This category included enlarged amastigotes, ellipsoid forms and promastigote-like cells but with short flagella equivalent to less than one cell body length. (iii) Multiplicative promastigotes, which were defined as promastigote-like cells with a flagellum of approximately one body length (thus distinguishing them from transformation intermediates) and not possessing metacyclic-like morphology. (iv) Metacyclic promastigotes, defined as short ($\leqslant 8{\cdot}4$ μm), narrow ($\leqslant 1{\cdot}5$ μm) cells with flagella greater than one cell body length (see Bates & Tetley, 1993 for a fuller description). (v) Type II transformation intermediates. After raising the temperature from 25 to 32 °C various intermediates in the transformation of metacyclics to amastigote-like forms were observed. These included cells with rounded cell bodies and flagella of various lengths down to a short but detectable stump. (vi) Aflagellates, which were defined as rounded cells ($\leqslant 5$ μm) lacking any detectable emergent flagellum. A minimum of 200 cells were examined and classified for each time-point.

Protein determination

Preparation of cell lysates and determination of protein content was performed as described (Bates, 1993*b*).

Gelatin SDS–PAGE

Proteinases with activity against gelatin were analysed by SDS–PAGE as described (Lockwood *et al.* 1987; Robertson & Coombs, 1990). Briefly, this involved inclusion of 0·2% (w/v) gelatin in the resolving gel, standard electrophoretic conditions and renaturation of enzyme activity in the gel after electrophoresis. Samples were neither reduced nor heated prior to electrophoresis. Activity against gelatin was revealed by staining with Coomassie Blue, which indicated the positions of enzymes as clear bands within a blue-staining gel.

Nuclease SDS–PAGE

Nucleases with activity against polyadenylic acid (poly(A)) were analysed by SDS–PAGE as described (Bates, 1993*b*). Briefly, this involved inclusion of 0·3 mg/ml poly(A) in the resolving gel, standard electrophoretic conditions and renaturation of enzyme activity in the gel after electrophoresis.

Samples were heated in a boiling water bath for 2 min but were not reduced prior to electrophoresis. Activity against poly(A) was revealed by staining with Toluidine Blue, which indicated the positions of enzymes as clear bands within a blue-staining gel.

Acid phosphatase

Extracellular acid phosphatase (EC 3.1.3.2) activity was assayed using the substrate *p*-nitrophenyl phosphate as described (Gottlieb & Dwyer, 1982). One unit of enzyme activity is equivalent to the hydrolysis of 1 μmole of *p*-nitrophenyl phosphate/min at 37 °C. Cell-free culture supernatants were prepared by centrifugation of cultures at 2000 *g* and 4 °C for 10 min and filtration of the resulting supernatant medium through 0·22 μm Millex GV filters (Millipore).

RESULTS

To reproduce an entire developmental sequence for *L. mexicana* in axenic culture previous methods (Bates *et al.* 1992; Bates & Tetley, 1993) were combined and the following protocol developed (Fig. 1A). On day 0, lesion amastigotes were isolated from infected CBA mice, inoculated at 5×10^5/ml into HOMEM supplemented with 10% (v/v) foetal calf serum (FCS) at pH 7·5, and incubated at 25 °C. Under these conditions the amastigotes transformed into promastigote forms within 24–48 h and began to multiply. At the late logarithmic phase of growth on day 3, the resulting promastigotes were subpassaged into Schneider's *Drosophila* medium supplemented with 20% (v/v) FCS at pH 5·5 and incubated at 25 °C. This medium induced metacyclogenesis as previously described (Bates & Tetley, 1993) and the resulting stationary phase population on day 9 contained a homogeneous population of metacyclic forms. These were then subpassaged into the same medium, but the temperature of incubation was increased to 32 °C. These are conditions which support long-term axenic growth of amastigote-like forms (Bates *et al.* 1992). This subpassage resulted in transformation of the metacyclics to amastigote-like forms which subsequently multiplied in axenic culture until they reached stationary phase at day 16. Thus a complete developmental sequence, initiated with lesion amastigotes, proceeding through multiplicative promastigotes, metacyclic promastigotes and finally to amastigote-like forms, was obtained. This experiment has been performed on 6 separate occasions with essentially identical results. Each of the analyses of the resulting parasites described below in Figs 2–6 have also been performed on at least 2 separate occasions. However, for the sake of comparison, the actual data shown in Figs 1–6, which are representative of this larger number of experiments, were obtained from a single series of cultures upon which all the different types of analysis were performed.

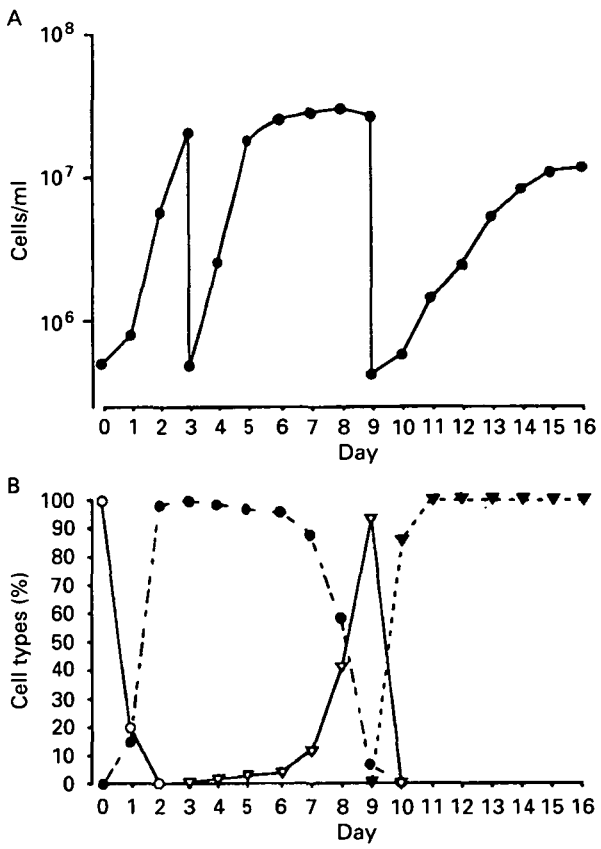

Fig. 1. Sequential axenic culture of *Leishmania mexicana* multiplicative promastigotes, metacyclic promastigotes and axenic amastigotes. (A) Cell density: the vertical lines indicate subpassage on days 3 and 9. (B) Percentage cell types: (○) lesion amastigotes; (●) multiplicative promastigotes; (▽) metacyclics; (▼) axenic amastigotes. For the sake of clarity, type I and type II transformation intermediates, present on days 1 and 10, respectively, are not shown.

The relative percentages of amastigotes, multiplicative promastigotes and metacyclic promastigotes were determined from Giemsa-stained smears and the results are shown in Fig. 1B. By day 1 the majority of the initial population of lesion amastigotes had transformed to type I transformation intermediates (65%): a mixture of elongated amastigotes and promastigotes with short flagella. This was accompanied by an increase in cell density (*cf.* Fig. 1A) and intermediate forms were noted in the process of division (see Fig. 2). Amastigote to promastigote transformation was essentially complete by day 2 and resulted in a log phase population of multiplicative promastigotes which exhibited a variety of morphologies (see Fig. 2C–F). These were the predominant cell population from day 2 to day 7. Metacyclic-like promastigotes were first noted on day 4 but only at very low prevalence. By day 9, however, they represented 95% of the cell population. These were then transformed to amastigote-like forms by incubation at 32 °C. The metacyclics

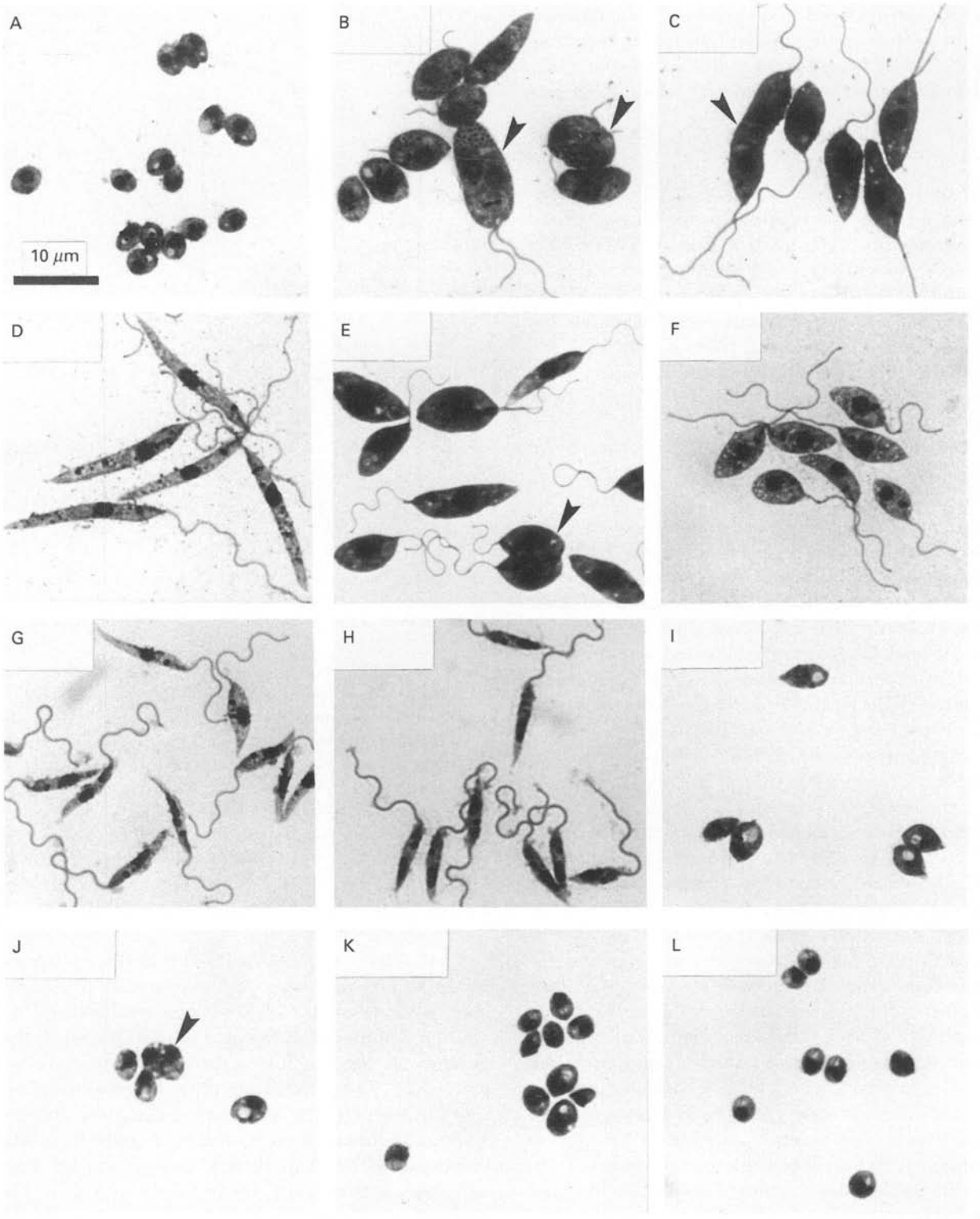

Fig. 2. Morphology of parasites in Giemsa-stained smears from various days of the developmental cycle *in vitro*. (A), day 0; (B) day 1; (C) day 2; (D) day 3; (E) day 4; (F) day 5; (G) day 7; (H) day 9; (I) day 10; (J) day 11; (K) day 13; (L) day 16. All pictures shown are at the same magnification.

resorbed their flagella and adopted a rounded morphology such that by day 10, 85% of the population were rounded aflagellates. However, from their morphology and biochemistry (see below) fully transformed amastigote-like forms were not present until day 11. The remaining 15% of cells present on day 10 were type II transformation intermediates. Amastigote-like forms accounted for 100% of the parasites observed from day 11 onwards.

The morphology of the parasites at various points in the developmental sequence is illustrated in Fig.

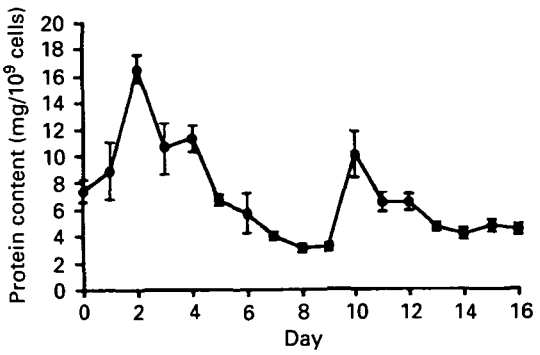

Fig. 3. Protein content of parasites through the developmental sequence. Estimates are the means from 4 determinations, error bars are 1 standard deviation.

2. The lesion amastigotes were small ovoid cells of mean body length $3{\cdot}86 \pm 0{\cdot}40$ μm on the major axis, and which lacked emergent flagella (Fig. 2A). Within 24 h most of these had elongated, developed short flagella and dividing forms were observed (arrowed, Fig. 2B). The resulting promastigotes continued to multiply and grow in size. The mid-log phase population on day 2 consisted of large broad promastigotes which stained very intensely with Giemsa's stain (Fig. 2C). By day 3 at the late log phase of growth the population consisted largely of elongated promastigotes with a mean body length of $15{\cdot}22 \pm 3{\cdot}49$ μm (Fig. 2D). These were then sub-inoculated into medium at pH 5·5 with the temperature remaining at 25 °C. The mid-log phase population on day 4 resembled those on day 2 in general appearance and intensity of staining (*cf.* Fig. 2C and 2E). However, the late log phase population on day 5, in comparison with those from day 3, were much shorter and fatter promastigotes with a mean body length of $8{\cdot}08 \pm 1{\cdot}39$ μm (*cf.* Fig. 2D and 2F). By day 7 the promastigotes had further reduced in size becoming both shorter and narrower as differentiation to metacyclic forms occurred (Fig. 2G). By day 9 a relatively homogeneous stationary phase population of metacyclic forms was produced with a mean body length of $7{\cdot}59 \pm 1{\cdot}15$ μm (Fig. 2H). When the temperature was raised to 32 °C these had transformed into aflagellates by day 10, that appeared slightly larger than amastigotes and were often ellipsoid in shape (Fig. 2I). Subsequently the parasites became amastigote-like (Fig. 2J) and grew axenically (Fig. 2K, cells $3{\cdot}99 \pm 0{\cdot}54$ μm) until stationary phase was reached (Fig. 2L, cells $3{\cdot}59 \pm 0{\cdot}42$ μm).

To determine whether the different cell populations were biochemically distinct various properties of parasites harvested on each day of the developmental sequence were analysed. The total protein content/cell is shown in Fig. 3. As lesion amastigotes transformed and grew as promastigotes an increase in protein content was observed, which rose from approximately 7 mg/10^9 cells on day 0 to 16 mg/10^9 cells in the mid log phase population on day 2. This correlated with an increase in size of the parasites as described above. Interestingly, by day 3 at the late log phase of growth the protein content had decreased somewhat coincident with the appearance of the elongated promastigotes, and this process continued upon subpassage at pH 5·5. The metacyclics on day 9 had an estimated protein content of 3·5 mg/10^9 cells, the lowest observed. Incubation at 32 °C resulted in a dramatic increase in protein content to approximately 10 mg/10^9 cells. On subsequent days this was observed to decrease such that the axenically growing amastigotes remained at approximately 5 mg/10^9 cells.

The proteinases expressed by parasites on each day of the developmental sequence were analysed by gelatin SDS–PAGE and the results shown in Fig. 4. The lesion amastigotes possessed a prominent group of bands of apparent molecular mass 22–24 kDa (arrowed, Fig. 4) which have been extensively characterized and previously shown to represent cysteine proteinases (Robertson & Coombs, 1990). They are also expressed by axenically cultured amastigotes of *L. mexicana* (Bates *et al.* 1992). These became undetectable in cell lysates of parasites harvested from days 2 to 5 as they transformed and grew as multiplicative promastigotes. However, in day 6 samples 2 bands were observed which increased in intensity until day 9, when the parasites were predominantly metacyclic promastigotes. A band equivalent to the faster migrating of these two bands was not detected in amastigotes. Upon transformation to axenically growing amastigotes this pattern was replaced by that observed in lesion amastigotes. This process appeared to be essentially complete by day 11 and the pattern remained constant thereafter in lysates of axenically cultured amastigotes.

The nucleases expressed by parasites harvested from each day were also analysed by substrate SDS–PAGE using poly(A) and the results are shown in Fig. 5. The lesion amastigotes possessed two prominent bands of apparent molecular masses 29 and 31 kDa as previously shown (Bates, 1993*b*). Upon transformation to promastigotes these decreased in intensity but did not disappear completely. However, an additional promastigote-specific band of 40 kDa was induced. This was detectable from day 1 through to day 9 but appeared to be expressed at the greatest specific activity on day 3, judging from the relative intensity of the banding pattern (Fig. 5). This band was lost upon transformation to amastigotes (day 10), coincident with a marked increase in the intensity of the 29/31 kDa doublet, thus restoring an amastigote-like pattern of enzymes from day 11 to day 16.

Secretory acid phosphatase in the supernatant culture medium of the parasites was assayed and the results are shown in Fig. 6. Extracellular enzyme

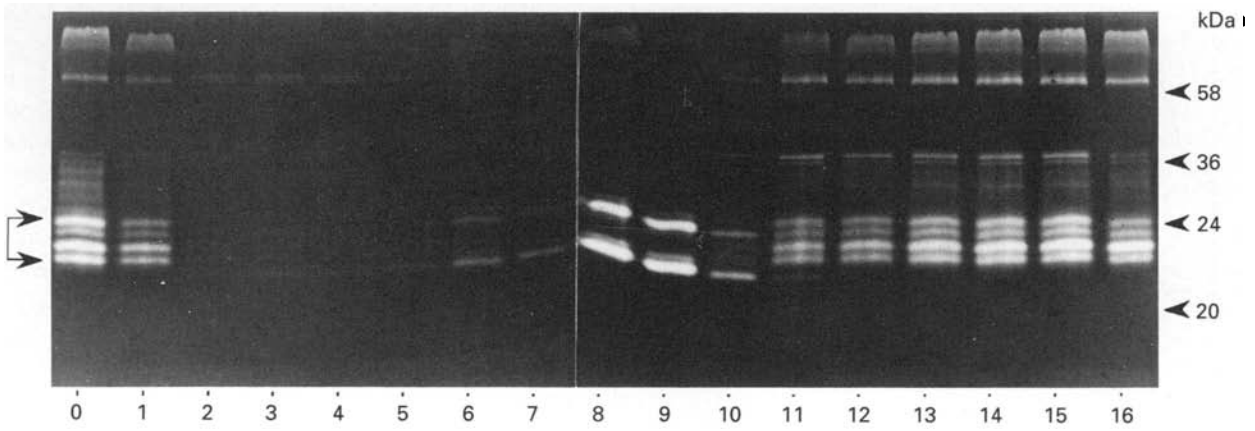

Fig. 4. Gelatin SDS–PAGE of parasite lysates from cells harvested through the developmental sequence (days 0–16). Molecular mass markers are: pyruvate kinase 58 kDa; glyceraldehyde-3-phosphate dehydrogenase 36 kDa; trypsinogen 24 kDa; soybean trypsin inhibitor 20 kDa.

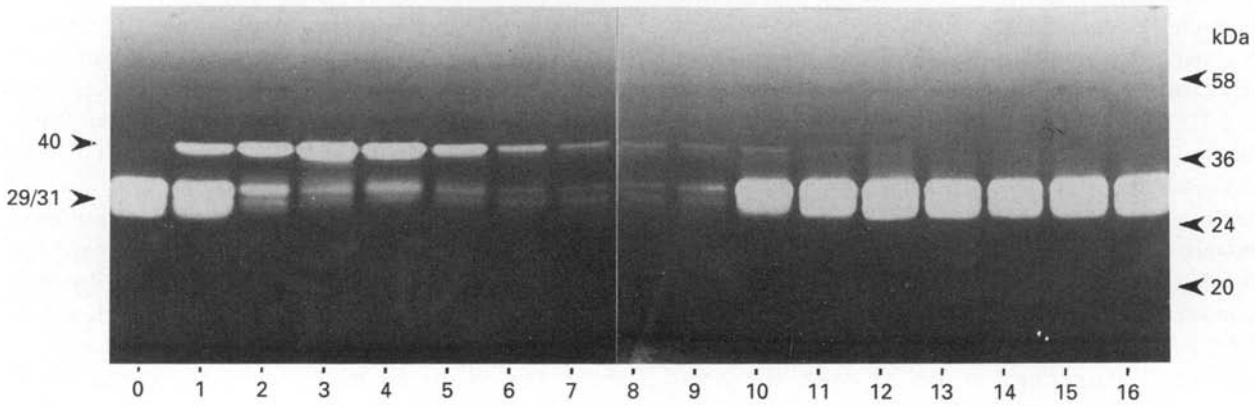

Fig. 5. Nuclease SDS–PAGE of parasite lysates from cells harvested through the developmental sequence (days 0–16). Molecular mass markers are as in Fig. 4.

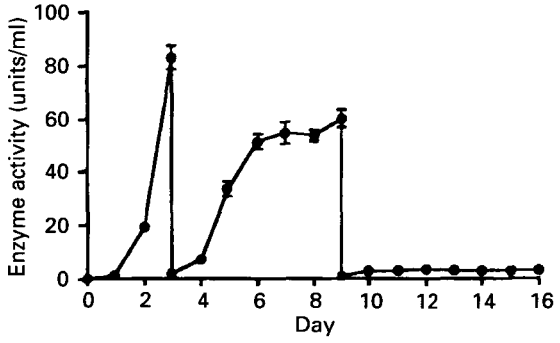

Fig. 6. Secretory acid phosphatase in cell culture supernatants through the developmental sequence. Estimates are the means from 3 determinations, error bars are 1 standard deviation.

activity rapidly increased as lesion amastigotes transformed and grew as promastigotes on days 1–3. This process continued upon subpassage at day 3 until day 6 but halted as the cultures entered stationary phase concomitant with the production of metacyclics. When the metacyclics transformed and grew as amastigote-like forms very little activity could be detected in the supernatant medium of axenically cultured amastigotes (days 10–16).

DISCUSSION

The current report is the first to demonstrate a complete developmental sequence for *Leishmania* in axenic culture, initiated with amastigotes and proceeding through multiplicative promastigotes, metacyclic promastigotes and returning to amastigotes. This was achieved by manipulation of culture conditions using changes in both pH and temperature and clearly demonstrated the importance of these environmental cues in regulating parasite growth and development. The resulting sequence of morphological forms resembled that observed in the natural life-cycle of the parasite. The experiments described were all initiated with lesion amastigotes, i.e. those derived from an infected animal, rather than from long-term cultures, because culture-adapted parasites are likely to differ significantly from their *in vivo* counterparts. For example, it is known that the long-term culture of promastigotes can result in a decreased capacity to produce metacyclic forms (Da Silva & Sacks, 1987).

Three main stages can be defined in the *Leishmania* life-cycle: multiplicative promastigotes, metacyclic promastigotes and amastigotes. In the current study

these correspond to *L. mexicana* populations from days 2–5, day 9 and days 0/11–16, respectively. Multiplicative promastigotes showed little detectable cysteine proteinase activity (Robertson & Coombs, 1992), exhibited a promastigote nuclease banding pattern, including a promastigote-specific band (Bates, 1993*b*), and also secreted acid phosphatase (Ilg *et al.* 1991). Metacyclic promastigotes were cultured as previously described (Bates & Tetley, 1993) and in the present study showed two prominent bands in proteinase gels. These have been observed previously and are only found in stationary-phase populations of *L. mexicana* promastigotes (Robertson & Coombs, 1992). They appear to be metacyclic-specific as there was a strong correlation between the prevalence of metacyclic promastigotes in cultures and the detection of these bands in the corresponding lysates when examined by gelatin SDS–PAGE (Bates, unpublished observations). Lesion amastigotes and axenically cultured amastigotes both showed appropriate morphology, and expressed amastigote-specific proteinases (Robertson & Coombs, 1990; Bates *et al.* 1992) and nucleases (Bates, 1993*b*). There was no evidence for secretion of acid phosphatase by amastigotes, consistent with short-term incubations of amastigotes (Antoine *et al.* 1987; Stierhof *et al.* 1991) and long-term cultures of amastigote-like forms (Russell, Xu & Chakraborty, 1992; Bates, unpublished observations). Thus, each of the individual populations exhibited morphological and biochemical properties consistent with previous studies.

In contrast to multiplicative promastigotes, metacyclics did not appear to secrete acid phosphatase, as the extracellular activity showed a plateau coincident with metacyclogenesis and entry into stationary phase. Similar effects have been noted before in populations likely to contain few, if any, metacyclics (Gottlieb & Dwyer, 1982) and this feature may, therefore, be a general property of stationary-phase promastigotes. The alternative explanation, that the plateau resulted from a balance between synthesis and degradation is less likely as there was no evidence of cell lysis here or previously (Gottlieb & Dwyer, 1982) and extracellular proteinase activity has not been detected (Bates, unpublished observations). Thus, secretion of acid phosphatase appears to be a stage-specific feature in *L. mexicana*, restricted to multiplicative promastigotes. In this regard, it has been previously shown that the flagellar pocket is the route for secretion in multiplicative promastigotes of *L. donovani* (Bates, Hermes & Dwyer, 1989) and *L. mexicana* (Stierhof *et al.* 1991). Monoclonal antibody staining indicated that although *L. mexicana* amastigotes do synthesize acid phosphatase, it is retained in the flagellar pocket (Stierhof *et al.* 1991). These observations support the idea that amastigotes attempt to minimize the exposure of their antigens to the immune system (Russell *et al.* 1992) and suggests that metacyclics, the mammal-infective form, have adopted a similar strategy. However, the lack of secretion cannot be simply explained as a non-specific blockage, as it is still possible for various tracers to gain access to the flagellar pocket in amastigotes (Russell *et al.* 1992).

In addition to providing an opportunity to compare the main life-cycle stages, the present study offers a unique system in which to examine the transitions between these stages. However, it should be emphasized that the morphological categories, whilst useful to illustrate the sequential appearance of the major forms, are essentially arbitrary divisions in a continuous sequence. The multiplicative promastigotes, in particular, exhibited variable morphology, both on a given day and between different days of culture. The functional significance of such variation is unknown but is partly explained by the existence of promastigotes at various points in the cell cycle. However, changes in morphology with time were also reported for development *in vivo* within sandflies in studies of *L. mexicana* (Lawyer *et al.* 1987; Walters *et al.* 1987) and other species. For example, in a study of *L. major* ten distinct morphologies were identified (Lawyer *et al.* 1990). In this respect, the possible reasons for the change from the broad promastigotes observed on day 2 to the elongated forms observed on day 3 are of interest. These two populations bear close resemblance to the promastigotes which initially multiply in the bloodmeal and those which occur subsequently attached to the midgut epithelium (Lawyer *et al.* 1987; Walters *et al.* 1987). These have been termed procyclic and nectomonad promastigotes, respectively (Lawyer *et al.* 1990).

Interestingly, the protein content/cell showed a marked increase from day 9 to day 10, but then decreased again. This temporary increase occurred even though the cells were not obviously larger, although they were a different shape, but was also coincident with the increase in temperature of incubation from 25 to 32 °C. Therefore, this effect is probably explained by the induction of a protective heat shock response (reviewed by Maresca & Carratù, 1992) in addition to the induction of amastigote stage-specific proteins. The decrease observed from day 10 to day 11 may be explained by cell division and the turnover of heat shock proteins or promastigote-specific proteins as full biochemical transformation was completed.

The current study is the first to investigate metacyclic to amastigote transformation in axenic culture and permits a comparison with amastigote to promastigote transformation (Hart *et al.* 1981) to be made. In general outline the process of transformation is initiated when appropriate environmental signals are received by the parasites (for example, changes in temperature and/or pH) which, in turn, result in biochemical and morphological changes.

The main changes in morphology in the transformation of metacyclics to amastigote-like forms, resorption of the flagellum and rounding of the cell body, appeared to precede any significant increase in cell numbers. However, the day 10 aflagellates differed morphologically from true amastigote-like forms and, also from the biochemical results, fully transformed amastigotes did not appear until day 11, by which point the cell density was already increasing. Amastigote to promastigote transformation exhibited similar features. Thus, cells were found in the process of division before the morphological or biochemical changes were completed as shown here and previously (Hart *et al.* 1981). Therefore, it appears that morphological and biochemical changes accompany division in both kinds of transformation. Entry into the cell cycle is initiated from an arrested state in the case of metacyclics, which are a non-dividing cell population. The growth status of lesion amastigotes is not well defined, although as an overall population lesion amastigotes are not growing as fast as their *in vitro* axenic equivalents. Thus, whether lesion amastigotes are a mixture of arrested and growing cells or a population with a much lower growth rate or some combination, is unknown, and may also vary with the age of and site within the lesion. Also the possibility that a differentiated amastigote transmission stage exists, although a matter for speculation, should not be dismissed. In any event, for both amastigotes and metacyclics, cell division occurs during transformation to the next stage. It is an interesting possibility that cell division may be obligatory to achieve complete transformation, or alternatively the apparent linkage of these processes could simply result from a timing effect. Ultimately, the next stage is produced which then undergoes repeated cycles of growth and division to establish an infection in the new host. More detailed investigation of the time-course of events at the molecular level will be required to understand these processes more fully.

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